Production of Ligninolytic Enzymes by Species Assemblages of Tropical Higher Fungi from Ecuador

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Introduction

No systematic screening studies have been made on lignin-degrading enzymes produced by higher fungi isolated from tropical moist forests. This is a surprising anomaly, since net primary production in moist tropical forests exceeds that of any other terrestrial biome, with a litter production of 10–35 t ha^{-1} $year^{-1}$, of which wood can constitute 74% (Swift *et al.*, 1976). Assuming an average lignin content of 20%, the annual input of wood lignin is thus 1.5–5.3 t lignin ha^{-1}. The decomposition constant, k value, for moist tropical forest litter is high, estimated by Swift *et al.* (1976) at ~6.0, underlining the rapid turnover of lignocellulose in the ecosystem. Reviews, such as, by Jordan (1985) that have emphasized that fungi and termites are responsible for most of this decomposition.

The mechanisms of lignin degradation by fungi involve a variety of enzymes, including polyphenol oxidase (laccase) and the peroxidative enzymes, such as lignin-peroxidase and manganese-dependent peroxidase (Kirk and Farrell, 1987; Thurston, 1994). Lignin is one of the three major polymers in wood cell walls, which provide strength and rigidity, the others being cellulose and hemicellulose.

White rot fungi are considered to cause degradation of all three polymers, either as simultaneous or as sequential rots, leaving some of the white hemicellulose and cellulose behind (Kirk *et al.*, 1978; Evans *et al.*, 1991). In contrast, brown rots are considered to selectively degrade the cellulose and hemicellulose components, possibly by non-enzymic peroxide oxidation and with oxalic

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acid–hydrogen peroxide, leaving the lignin behind as a modified brown-coloured polymer (Kirk and Fenn, 1982; Dutton *et al.*, 1993). White rot fungi predominate in moist tropical forest, especially members of the *Agaricales*, aphyllophoroid taxa and *Xylariaceae*, but brown rot fungi are almost completely absent, possibly because they co-evolved with the *Coniferae* in the boreal forest biome (Watling, 1982). No detailed studies on the ligninolytic enzymes produced by tropical wood, white rot fungi are known, or on the other major group of fungal decomposers in rainforests, the litter-degrading fungi. The readily observed bleaching of forest floor leaf litter, which is caused by the mycelia of such fungi, indicates that exploitation must also involve degradation of lignin. However, the physico-chemical environment of the small litter resources is very different from that of larger volumes of wood, and could influence the types of ligninases used to depolymerize them.

Accordingly, it was thought that a survey of the ligninolytic systems found in the species assemblages of fungi on these two resource types, wood and litter, would provide much needed data on the influence of resource selectivity on the exploitative enzymes produced by the fungi.

Methods

Study site

The fungal cultures used in the study, apart from *Trametes versicolor* Quélet (FPRL-28A supplied by CABI, Egham, UK), had been isolated from Rio Palenque, a 87 ha reserve located on the west side of the Cordillera in Ecuador some 65 km south of the Equator, at an altitude of 150 m. The flora has been fully described by Dodson and Gentry (1978). It consists of primary Pluvial Pacific Coastal forest, with a very high species diversity, and of disturbed forest rich in gap colonizing trees such as balsa (*Ochroma pyrimidale* (Cav : Lam.) Urb.) together with extensive clumps of bamboo (*Bambusa guadua* H. & B.). An account of the ecology of decomposer fungi in this zone of Ecuador is given in Hedger (1985) and of the Amazonian forest in Ecuador by Lodge and Cantrell (1995).

Survey of fruit bodies

The survey of the resource relations of higher fungi in Rio Palenque was carried out by random fruit body collection in September 1997. Over 100 collections were made. Two resource types were distinguished: wood and small litter. Wood included all parts of fallen and standing dead, or partially dead, trees, down to branches > 20 mm in diameter, while small litter included twigs < 20 mm in diameter, leaf litter and reproductive structures. The resource component on which each fruit body occurred and the state of decay immediately beneath it was noted, in order to infer the resource relationships of the mycelium (Rayner *et al.*,

1985; Frankland, 1992). This information was used to group the taxa into resource-based species assemblages, and was combined with an existing database obtained from similar surveys carried out previously in Rio Palenque, between 1981 and 1996, in which 793 collections of macrofungi were made (J.N. Hedger, London, unpublished data). The authors prefer the use of the term species assemblage to community, since the fruit body distribution could not provide implicit evidence that mycelia of these taxa were part of an interactive fungal community.

Isolation of mycelia

Isolations were carried out in the field in September 1997, using a portable Perspex isolation cabinet, which could be sterilized by swabbing out with alcohol. Portions of fruit bodies were inoculated on to pre-poured selective media in 2.5 cm diameter plastic Petri dishes, using a spirit lamp to flame the isolation tools, and sealed with Parafilm. For agarics, a spore-fall method was also used, in which a pileus was attached to the lid of a Petri dish with sterile Vaseline, left to deposit basidiospores overnight on to the medium and removed the next morning. Members of the *Sarcoscyphaceae* and *Xylariaceae* were isolated from ascospores discharged from portions of the ascomes stuck on the lids of Petri dishes containing media and incubated upside down. The media used were selective for basidiomycetes or ascomycetes and are described by Hedger (1982). After incubation at room temperature (20–29°C) for 24–36 h, in a sealed plastic bag to prevent entry of ants, the plates were checked from below for presence of clamp connections (basidiomycetes) and ascospore germination and hyphal morphology (ascomycetes). Inocula were then transferred in the laboratory to 3% malt agar (MA) or 2% potato dextrose agar (PDA) slants and grown at 26°C; plugs of these mycelial cultures were stored in crio-vials under sterile water at 20°C. The fungi were recovered by transferring inoculum on to the centre of Petri plates of the same medium, and incubated for 7 days at 26°C, before checking hyphae for purity. For screening studies, 4 mm diameter inocula marked out with a sterile cork borer were removed from edges of 3–7-day-old, 4–6 cm diameter colonies, growing on 3% MA or 2% PDA plates at 26°C.

Screening for ligninolytic enzymes

Ten taxa from each species assemblage were screened for ligninolytic enzyme production in liquid culture. The basis of the choice is described later. Measurements showed that, in cultures in which ligninolytic enzyme activity had been induced, these enzymes represented up to 90% of total protein in the culture filtrate. Activity measurements are presented as activity units per mg dry wt mycelial biomass, which was considered to be a more realistic comparative measure of ligninolytic activity of the isolates than specific activity of the protein fraction of the culture filtrates.

Laccase production

For laccase production, a liquid glucose–amino acid–salts medium was used (Fahraeus and Reinhammar, 1967). Conical flasks (50 ml) containing 25 ml of medium were inoculated in triplicate with 4 mm × 4 mm diameter mycelial plugs for each organism. The flasks were shaken at 26°C for 7 days, when 2,5-xylidine was added 18 h before harvesting to give a concentration of 2×10^{-4} M in the growth medium (Fahraeus and Reinhammar, 1967). Mycelium was then separated from the medium by filtration and samples of 5 – 20 μl culture filtrate were removed and added to 1.0 ml of catechol (10 mM) in 100 mM sodium acetate buffer at pH 5, and laccase activity measured by increase in absorbance at 440 nm (Evans and Palmer, 1983). One unit of laccase was defined as causing a change in absorbance of 1.0 ml^{-1} min^{-1} at 25°C, each value being the mean of three replicate cultures.

Manganese peroxidase (MnP) production

For MnP production, static cultures were prepared in 300 ml medical flats containing 50 ml of low-nitrogen medium adapted from Glenn and Gold (1985). The medium contained glucose (20 g l^{-1}), ammonium tartrate (1.2 mM), mineral salts and vitamins. The mineral salts (g l^{-1}) included 0.01 $CaCl_2$, 0.01 $FeSO_4 . 7H_2O$, 0.001 $MnSO_4 .4H_2O$, 0.001 $ZnSO_4 .7H_2O$, 0.002 $CuSO_4$. The vitamin solution (mg l^{-1}) included 27.5 adenine, 0.05 thiamine HCl, 1000 *I*- inositol, 100 pyridoxine-HCl, 100 nicotinic acid, 100 sodium pantothenate, 100 *p*-aminobenzoic acid, 100 riboflavine, 30 biotin, 10 folic acid, 10 cyanocobalamin. The flasks were incubated statically for 14 days at 26°C, and MnP activity was measured by the transformation of 100 μM of 2,6-dimethoxyphenol (DMP) to coerulignone (Martinez *et al.*, 1996). The reaction mixture included 100 μM $MnSO_4$ in 100 mM sodium tartrate buffer at pH 5.0 and 100 μM H_2O_2 with 5–20 μl enzyme extract to be assayed. Absorbance was measured at 238 nm with a molar extinction coefficient of 6500 M^{-1} cm^{-1} and one unit of MnP was equivalent to one mole of coerulignone formed under reaction conditions (c = A/E), each value being the mean of three replicate cultures.

Results

Species assemblages of fungi in Rio Palenque

The analysis of the 1997 data and those of previous years showed that the two major resource-related species assemblages of saprobic fungi in Rio Palenque, on small litter and on wood, together accounted for over 90% of the collections. Fungi on soil and arthropods together made up only around 10% of the

collections. No obviously mycorrhizal taxa were found, except for one unidentified species of *Boletus* in 1982.

The species assemblages on small litter and wood, respectively, accounted for approximately 30% and 60% of the macrofungi surveyed. A number of taxa were common to both of these assemblages, especially the species found both on small litter and on the smaller woody resources, branches 20–40 mm diameter. However the surveys showed that this species overlap was only ~20% of the total, 80% of the taxa fruiting exclusively on either wood or small litter. There was no overlap at all between the species fruiting on small litter and those present on the larger woody resources, branches > 50 mm diameter and tree trunks. Lodge (1997) also found, in a resource survey of rainforest fungi in Puerto Rico, that only 173 (25%) of the 705 decomposer fungi she recorded occurred on two resource types, whereas 493 taxa (70%) occurred on only one resource type.

Ten isolates were selected from each species assemblage for further study of ligninolytic enzymes. The basis of selection was to attempt to sample a broad spectrum of mycelial behaviour from within each assemblage, including different decomposition strategies and differences in component and taxon selectivity. The fungal taxa selected are shown in Table 8.1, arranged with respect to their species assemblages. Also included in this table is a field assessment of the state of decomposition of the resource on which the fruit bodies were found, although whether the mycelia of these taxa had caused this decomposition could not be proved.

The mycelia of all fungi on small litter caused bleaching or white rot of the litter material, implying that they were capable of simultaneous degradation of lignin, hemicellulose and cellulose. Much variation in component and taxon selectivity operated within this assemblage, related to the mixture of available resources (leaves, twigs, fruits, seeds and other plant debris). Some taxa were foliicolous, almost entirely restricted to leaves. The genus *Marasmius* exemplified this strategy, and many species appeared to be component- and taxon-selective. The mycelia of these species could be easily identified as discrete patches of bleaching, always on intact leaves in the upper litter layer on the soil surface, and also on leaves trapped in the understorey shrubs. However the identities of the leaves on which each *Marasmius* species occurred were difficult to establish. *Marasmius cladophyllus*, which was readily observed to be taxon-selective for leaves and petioles of *B. guadua*, was isolated for further study. *Favolaschia cinnabarina* was also chosen, because it was obviously component-taxon selective for petioles of palm and *Heliconia* species. Other taxa isolated, such as *Collybia bakeri*, *Xeromphalina tenuipes*, and *Lepiota lactea* had non-component-selective mycelia, which advanced as a front colonizing all litter components. Four aphyllophoroid taxa, *Polyporus obolus*, *Polyporus lepreurii*, *Trametes ochracea* and *Coltricia spathulata* were also chosen, since they were clearly component-selective decomposers, occurring only on small twigs and branches at soil level, which they bound together by pseudosclerotial plates (psps; Ainsworth and

Table 8.1. Fungal cultures selected for ligninolytic enzymes studies from two species assemblages studied in Rio Palenque forest.

Small litter assemblage	Family	Rot type
Collybia bakeri Dennis	*Tricholomataceae*	B
Lepiota lactea Murrill	*Agaricaceae*	B
Marasmius cladophyllus Berk.	*Tricholomataceae*	B
Xeromphalina tenuipes (Schwein.) A.H.Sm.	*Tricholomataceae*	B
Favolaschia cinnabarina (Berk. & M.A Curtis) Pat.	*Tricholomataceae*	WR
Nothopanus eugrammus (Mont.) Singer	*Tricholomataceae*	WR
Coltricia spathulata (Hooker) Murrill	*Hymenochaetaceae*	WR
Polyporus obolus Ellis. & T. Macbr.	*Polyporaceae*	WR
Polyporus lepreurii Mont.	*Polyporaceae*	WR
Trametes ochracea (Pers.) Gilb. & Ryvarden	*Coriolaceae*	WR
Lignicolous assemblage		
Cookeina tricholoma (Mont.) Kuntze	*Sarcoscyphaceae*	WR
Irpex maximus Mont.	*Steccherinaceae*	WR
Lentinus crinitus (L.) Fr.	*Lentinaceae*	WR
Lentinus strigosus (Schwein.) Fr.	*Lentinaceae*	WR
Stereum insigne Bres.	*Stereaceae*	WR
Trametes corrugata (Pers.) Bres.	*Coriolaceae*	WR
Xylaria guyanensis (Mont.) Mont.	*Xylariaceae*	WR
Psathyrella albocapitata Dennis.	*Coprinaceae*	WR
Filoboletus gracilis (Klotzsch : Berk.) Singer	*Tricholomataceae*	SR
Tomentella lilacinogrisea Wakef.	*Thelephoraceae*	?WR

B, bleaching of leaf litter by mycelium; WR, white rot of wood; SR, soft rot of wood.

Rayner, 1990; Hedger *et al.*, 1993). In the case of *P. lepreurii*, 2–4 mm diameter negatively gravitropic rhizomorphs were also produced. It is probable that this fungus was also taxon-selective, since its distribution in the forest was very disjunct. The sessile, tricholomatoid agaric *Nothopanus eugrammus* was also studied, since it appeared to have little component or taxon selectivity. It was most commonly found fruiting on twigs but also occurred on larger diameter tree branches.

In the lignicolous species assemblage, the wood below the fruit bodies was usually partially or completely bleached, sometimes with discoloration by pigments and psps, typical of fungal white rot attack. Association between fruit bodies and the mycelia in the decomposed wood below was investigated by cutting into the wood, using indicators such as relative wood bleaching, pigment production, odour and psps to determine that the mycelium in the wood had given rise to the fruit body. These decomposers could be divided on component selectivity and mode of attack on the wood. On fallen trunks, on the drier upper portions of the logs, there were large fruitings of many obvious decomposers. Examples of basidiomycetes selected for study were *Irpex maximus*, *Lentinus crinitus*, *Lentinus strigosus* and *Psathyrella albocapitata*, all of which were asso-

ciated with softened, moist, bleached wood. The ascomycetes *Xylaria guyanensis* and *Cookeina tricholoma* were also included in the study. Their ascomes, and those of other *Xylariaceae* and *Sarcoscyphaceae,* were associated with bleached wood which was hard and dry, with surface and internal psps present. On the wetter undersides of the logs the dominant decomposers were resupinate aphyllophoroid taxa, the example selected being *Stereum insigne*. *Tomentella lilacinogrisea* was also identified growing in this situation and its fruit bodies seemed to be associated with a white rot mycelium. However, A.M. Ainsworth (Slough, UK, personal communication) opines that this fungus is mycorrhizal, so that the association of mycelium with fruit bodies may have been incorrect in this instance. Its ecological status in Rio Palenque thus remains uncertain. Finally, *Filoboletus gracilis* was selected as a representative of soft rot attack. This fungus was always found on wood semi-immersed in small streams in the forest, and associated with grey, water-soaked, highly softened wood.

Production of ligninases

Figures 8.1 and 8.2 show the results of screening the twenty isolates for MnP and laccase activity. The results are grouped according to species assemblage, rather than taxonomic affinities.

Manganese peroxidase (MnP) activity in the species assemblages

The results of MnP assays of the culture filtrates are shown in Fig. 8.1. Although the results were variable, average values for the enzyme units of the two species assemblages showed an opposite trend to the laccase units, being significantly lower in the lignicolous assemblage, 4.3 units, than in the small litter assemblage, 12.5 units (t-statistics = 1.777, P = 0.05). Five of the small litter assemblage taxa had MnP titre values > 10 units in the culture filtrates (*C. bakeri*, *M. cladophyllus*, *P. obolus*, *P. lepreurii* and *T. ochracea*), while only one taxon in the lignicolous assemblage, *I. maximus*, had a value > 10 units. However, both species assemblages had three isolates showing zero or negligible MnP activity, *L. lactea*, *X. tenuipes* and *N. eugrammus* in the small litter assemblage and *L. crinitus*, *L. strigosus* and *X. guyanensis* in the lignicolous assemblage. The comparative figure for *T. versicolor* was 14.6 units.

Laccase activity in the species assemblages

The laccase titres (Fig. 8.2) of fungi from the lignicolous species assemblage showed a significantly higher average level of laccase, 284.8 units (t-statistics = 2.16, P = 0.05), than the mean for the taxa from the small litter species

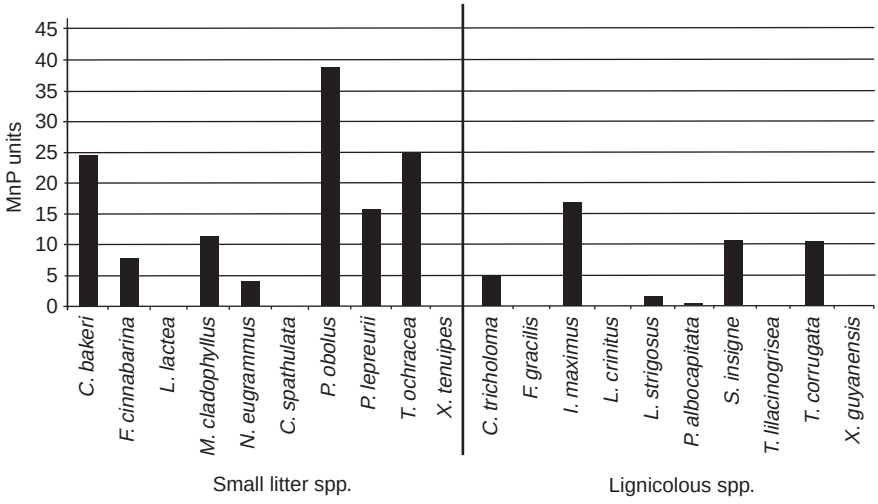

Fig. 8.1. Manganese peroxidase (MnP) activity of culture filtrates. One unit of MnP was equal to one mole of coerulignone formed under reaction conditions, using 100 μM of 2,6-dimethoxyphenol (DMP), each value being the mean of three replicate cultures.

assemblage, 6.4 units. Two of the small litter assemblage taxa had no detectable laccase in the culture filtrates (*N. eugrammus* and *T. ochracea*), and in *M. cladophyllus*, *C. bakeri* and *X. tenuipes* activity was barely detectable. Although the other five litter taxa had some laccase activity in their culture filtrates, the titre was much lower than that of the lignicolous species, the highest being found in the filtrates from *L. lactea* (18.2 units). In contrast, eight of the lignicolous species had a titre above 10 units, five had activities in excess of 100 units and *I. maximus* and *L. crinitus* had activities of more than 1000 units. Of particular interest were the two cultures from the lignicolous species assemblage which had titres lower than 10 units, *C. tricholoma* (5.4), and *X. guyanensis* (0), indicating there was little, if any, laccase expression by these fungi. The comparative laccase titre for *T. versicolor* was 1802 units.

The results in Figs 8.1 and 8.2 also show that two taxa, *X. guyanensis* and *X. tenuipes*, had zero, or near zero, values for both ligninolytic enzymes in their culture filtrates. Both taxa grew well on the culture medium and field data showed that both caused very active *in vivo* degradation of lignocellulose, *X. guyanensis* of wood and *X. tenuipes* of litter.

Discussion

The production of ligninolytic enzymes by these tropical fungi can be compared with the resource selectivity of their mycelia in the field. The clearest association was between high laccase levels and the wood species assemblage. Laccase is considered to play a substantial role in the degradation of wood cell walls by

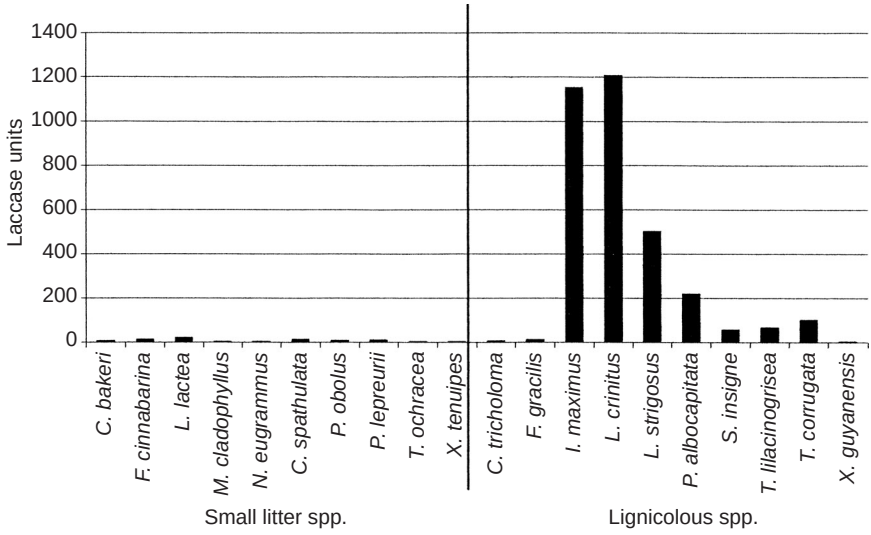

Fig. 8.2. Laccase activity in culture filtrates. One unit of laccase was defined as causing a change in absorbance of 1.0 min^{-1} ml^{-1} at 25°C, each value being the mean of three replicate cultures.

first attacking phenolic groups on lignin polymers, so generating free radicals for depolymerization reactions to occur, ultimately leading to the exposure of cellulose and hemicellulose to attack by removal of lignins from the s1 and s2 layers of the cell wall (Evans *et al.*, 1994). The data confirmed the importance of the enzyme in these tropical lignicolous fungi. The low laccase levels found in the litter species are less easy to understand. Attack on leaf litter and small branches by these fungi resulted in bleaching, usually followed by conversion to powder, indicating rapid simultaneous removal of lignin, cellulose and hemicellulose. It is true that the proportion of secondarily thickened wall material in the small litter resource must be lower than in wood (Jordan, 1985; Rayner and Boddy, 1988) and the few studies on the decomposition of the two resource types in tropical forest showed that the *k* values of wood are much lower than those of small litter (Swift *et al.*, 1976). However, it might be expected that laccase production by these litter decomposer fungi would be necessary for attack on the significant amount of lignin present, especially in the small twigs and branches. However, even the litter fungi that were component-selective for lignin-rich twigs and leaf petioles, the four aphyllophoroid taxa and *F. cinnabarina*, had a low laccase titre.

On the other hand, the higher MnP titre of the small litter species assemblage raises the possibility that this ligninase is more important to fungi in resources with a lower lignin content or a more diffuse distribution of lignified tissue. Culture conditions in the laboratory were optimized for fungal production of MnP and laccase to demonstrate the maximal capability of the organism

for enzyme production. Under these conditions of optimized production more laccase was produced in the wood assemblage species and more MnP in the litter assemblage. Ligninolytic enzyme activity depends on the substrate, the co-reactants (H_2O_2/O_2), and the pH of the environment. Both laccase and MnP react with phenolic lignin groups, although under some conditions they can attack non-phenolic moieties when appropriate mediators are present (Leontievsky *et al.*, 1997). Wood contains a larger proportion of lignin (~15–20%) than is present in litter (~5–10%) (Rayner and Boddy, 1988). More enzyme would therefore be required to degrade the wood lignin than the small-litter lignin and enable the fungus to gain access to the cellulose and hemicellulose in the resource. Increasing production of ligninases to meet this need may be more readily met by increased synthesis of laccase than MnP. In temperate species of fungi, induction of neutral laccases can be stimulated by addition of Cu^{2+} as in *Pleurotus ostreatus*, and acidic laccases by lignin mimics, such as 2,5-xylidene in *T. versicolor* (Palmieri *et al.*, 2000). The availability of oxygen for laccase activity is unlikely to be a metabolic block in the two resources, although it is probably lower in the wood resource (Hintikka, 1982), but hydrogen peroxide, a co-reactant for MnP, may be more available to the species of the litter assemblage due to the higher O_2 and more effective hydrogen peroxide production systems.

Perhaps the most likely reason for greater laccase production by wood decomposers and MnP production by litter decomposers is the environmental pH. The pH in wood is around 4, while the usual pH of litter in tropical forest is ~5–6 (Swift *et al.*, 1976; Rayner and Boddy, 1988). In the laboratory, the optimum pH for enzyme activity for laccase is 4.0–5.0, with most phenolic substrates, and for MnP pH 5–6. It is therefore possible that production of laccase by the tropical lignicolous fungi is an adaptation to a surrounding pH more suitable for optimizing its activity, while MnP is produced by tropical litter fungi in an environment where pH is higher. If enzymes are produced that have pH optima close to the environment in which the mycelium is growing, then less enzyme is required, thus reducing the amount of protein synthesis required by the fungus, an important factor in the nitrogen-poor environment of wood and litter in tropical forest, where competition for available nutrients is severe (Lodge, 1993).

The search for ligninase enzyme systems has concentrated on fungi almost wholly selected from the temperate, wood decomposer community and especially on just a few taxa, such as *T. versicolor*. This study of tropical higher fungi indicates that the ligninase systems produced by a fungal taxon are related to the type of resource exploited by the mycelium, so that wider community-focused studies on the fungi of wood, litter and soil resources may reveal different balances of laccase, MnP and perhaps novel ligninase enzyme systems than current models.

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